Induction of L-form-like cell shape change of *Bacillus subtilis* under microculture conditions

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A remarkable cell shape change was observed in *Bacillus subtilis* strain 168 under microculture conditions on CI agar medium (Spizizen's minimal medium supplemented with a trace amount of yeast extract and Casamino acids). Cells cultured under a cover glass changed in form from rod-shaped to spherical, large and irregular shapes that closely resembled L-form cells. The cell shape change was observed only with CI medium, not with Spizizen's minimum medium alone or other rich media. The whole-cell protein profile of cells grown under cover glass and cells grown on CI agar plates differed in several respects. Tandem mass analysis of nine gel bands which differed in protein expression between the two conditions showed that proteins related to nitrate respiration and fermentation were expressed in the shape-changed cells grown under cover glass. The cell shape change of CI cultures was repressed when excess KNO3 was added to the medium. Whole-cell protein analysis of the normal rod-shaped cells grown with 0–1% KNO3 and the shape-changed cells grown without KNO3 revealed that the expression of the branched-chain 2-keto acid dehydrogenase complex (coded by the *bfmB* gene locus) was elevated in the shape-changed cells. Inactivation of the *bfmB* locus resulted in the repression of cell shape change, and cells in which *bfmB* expression was induced by IPTG did show changes in shape. Transmission electron microscopy of ultrathin sections demonstrated that the shape-changed cells had thin walls, and plasmolysis of cells fixed with a solution including 0–1 M sucrose was observed. Clarifying the mechanism of thinning of the cell wall may lead to the development of a new type of cell wall biosynthetic inhibitor.

INTRODUCTION

Cell shape is one of the important characteristics used for the classification of bacteria. However, viable cell shape changes have been observed or induced by culture conditions, indicating that cell shape is not invariant for organisms. Conversion to a cell-wall-deficient form called ‘L-form’, which was first reported in 1935 for *Streptobacillus moniliformis* (Klieneberger, 1935), has been well documented for many species of bacteria. Because of the lack of a rigid cell wall, L-form cells generally show various shapes, for instance, small, large, spherical, irregular and club-shaped (Mattman, 2001a).

The L-form state has been observed and well characterized in the rod-shaped bacterium *Bacillus subtilis* (Burmeister & Hesseltine, 1968; Wyrick et al., 1973; Gilpin & Patterson, 1976; Gilpin et al., 1981). Gilpin et al. (1973) characterized the stable L-form of *B. subtilis* strain 168 morphologically and biochemically. They isolated a stable L-form mutant

Abbreviations: BCDH, branched-chain 2-keto-acid dehydrogenase; TEM, transmission electron microscopy.

A table of proteins detected by LC/MS/MS in cells grown in microculture on CI and CI+KNO3 is available as supplementary data with the online version of this paper at http://mic.sgmjournals.org.
and demonstrated that the L-form cells lacked a cell wall by electron microscopic observation and the analysis of incorporation of isotope-labelled dianimopimelic acid as a cell wall component. They also showed that there was a difference between the membrane proteins of the bacillary form and the L-form.

Regarding cell shape change in *B. subtilis*, recent molecular genetic studies have revealed that mutations in genes associated with cell envelope synthesis resulted in morphological change, as expected. In a study using an IPTG-inducible mutant, Henriques et al. (1998) demonstrated that the *rodA* gene, which controls peptidoglycan synthesis, is essential for growth and rod shape maintenance. The defect in teichoic acid synthesis also results in morphological change (Honeyman & Stewart, 1989; Máuel et al., 1989). In addition, it was reported that defects in MreB or Mbl proteins, which are assumed to form helical, actin-like filaments, lead to morphological change (Abhayawardhane & Stewart, 1995; Jones et al., 2001).

The existence of cell wall structure is one of the major differences between bacteria and animals; accordingly the inhibition of cell wall synthesis of bacteria is an important mode of action of antibiotics, and a large number of cell wall biosynthetic inhibitors have been developed. Here we report that *B. subtilis* cells grown under cover glass show a remarkable L-form-like morphological change, and describe changes in the whole-cell protein profile associated with this phenomenon. Mutants were studied to investigate the possible involvement of the *bfmB* locus in the changes in cell shape.

**METHODS**

**Bacterial strains and cultures.** The bacterial strains and plasmids used in this study are shown in Table 1. The media used in this study were: Spizizen’s minimal salts (1·4 % K2HPO4, 0·6 % KH2PO4, 0·2 % (NH4)2SO4, 0·1 % trisodium citrate dihydrate, 0·02 % MgSO4·7H2O, 0·5 % d-glucose) with 50 μg l-tryptophan ml⁻¹; CI (Spizizen’s medium supplemented with 50 μg l-tryptophan ml⁻¹, 0·05 % yeast extract and 0·02 % Casamino acids); Luria–Bertani (LB); Mycoplasma (BBL); BHI (Difco); and TBAB (Difco) plate medium. All media contained 1·5 % agar. For the inducible mutant (LPDd) culture, 1 mM IPTG was added to the CI agar medium when needed. Microcultures were performed as follows. One loop of overnight-precultured cells in LB liquid medium (supplemented with 0·5 μg erythromycin ml⁻¹ or 5 μg chloramphenicol ml⁻¹ for the mutants) was inoculated on the surface of agar media. After the inoculation spot (approx. 5 mm in diameter) had air-dried for a few minutes, the spot was sealed with an 18 mm-square cover glass (Matsunami, Japan). The cover glass was pre-soaked in alcohol and flamed just before covering for sterilization. Plates were incubated at 37 °C.

**Culture observations.** Bacterial cell growth under cover glass was observed by phase-contrast microscopy (Iponaclony IS-2000) and optical microscopy (Olympus BX-40) without removing the cover glass. For high-magnification observation by optical microscopy, immersion oil was dropped on the cover glass. Phase-contrast and optical microscopic images were recorded with a CCD camera (Shimadzu, CCD-X2) connected to a computer system.

**Whole-cell protein preparation.** Cells from 18 h cultures were collected and used for protein analysis. At this time, CI microcultures contained many burst cells. Cells grown on plates were suspended in 10 mM Tris/HCl buffer (pH 7.4) and then pelleted by centrifugation (5000 g, 5 min). Since the shape-changed cells grown under cover glass could not be pelleted completely, they were collected using a 0·1 μm filter spin-column (Ultrafree-MC, Amicon). Collected cells were resuspended in 27 μl BS (Bacillus subtilis) lysis buffer (20 mM Tris/HCl containing 10 % sucrose, pH 8·0) and then incubated at 37 °C for 30 min following the addition of 3 μl 10 mg lysozyme ml⁻¹ and 3 μl 10 mM PMSF (serine protease inhibitor, Sigma). Lysed cell solution was mixed with SDS-PAGE conditioning buffer to a final volume of 60 μl and boiled for 3 min. Protein was quantified with the BCA Protein Assay Kit (Pierce), using bovine serum albumin (Sigma) as a standard. The amount of the proteins into each lane was adjusted to approximately 35 μg excluding lysozyme.

**Protein identification.** Whole-cell proteins solubilized by SDS boiling were resolved by SDS-PAGE (12·5 % polyacrylamide gel) in Tris/glycine SDS running buffer (Laemmli, 1970) and visualized by Coomassie brilliant blue R-250 (CBB) staining. After excision from CBB-stained gels, proteins were reduced by dithiothreitol, alkylated by iodoacetamide and then in-gel digested with trypsin (Roche), according to the method of Shevchenko et al. (1996). Resulting peptides were eluted from the gel with several changes of extraction buffer (70 μl 70 % acetonitrile/5 % formic acid) and concentrated by iodoacetamide and then in-gel digested with trypsin (Roche), according to the method of Shevchenko et al. (1996). Resulting peptides were eluted from the gel with several changes of extraction buffer (70 μl 70 % acetonitrile/5 % formic acid) and concentrated by evaporation. Peptides were diluted with 5 % formic acid and 50 % acetonitrile. We used liquid chromatography combined with tandem mass spectrometry (LC/MS/MS), as previously applied for the identification of *B. subtilis* spore proteins (Kuwana et al., 2002), to analyse samples, using an LC-Q Deca mass spectrometer (Thermoquest) coupled with a Magic 2002 microcapillary nanoflow liquid chromatograph (Michrom Bioresources, USA). Proteins were identified

<table>
<thead>
<tr>
<th>Strain or plasmid</th>
<th>Relevant characteristics</th>
<th>Reference or source</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>B. subtilis</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>168</td>
<td>trpC2</td>
<td>Pasteur stock</td>
</tr>
<tr>
<td>ddBFMB</td>
<td>trpC2 Δ(bkdA1–bkdA2–bkdB):: bgaB–cat</td>
<td>This study</td>
</tr>
<tr>
<td>LPDd</td>
<td>trpC2 lpd::pMutinNC</td>
<td>This study</td>
</tr>
<tr>
<td><strong>Plasmids</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>pMutinNT</td>
<td>EmR ApR lacZ lacI Pspac</td>
<td>Morimoto et al. (2002)</td>
</tr>
<tr>
<td>pDX-CAT</td>
<td>cat bgaB</td>
<td>This study</td>
</tr>
</tbody>
</table>
using the TurboSEQQUEST computer program (Thermoquest), a method to correlate tandem mass spectra of modified peptides to amino acid sequences (Yates et al., 1995) in the protein database.

Construction of a gene deletion mutant and an IPTG-inducible mutant. The deletion mutant of the bfnB gene locus, including three genes, bkdA1, bkdA2 and bkdB (see Fig. 6), was constructed with pDX-CAT, pDX-CAT was constructed by a slight modification of pDX-EM (Kadaya et al., 2002) (the Em' marker was replaced by a Cm' marker). The PCR-amplified DNA fragments of the lpd and bmrR genes were ligated into MCS1 and MCS2, respectively, of pMutinNC, and the bfnB gene was replaced by bgab-cat in the B. subtilis chromosome via a double-crossover event. The primers used for PCR amplification were as follows: 5'-CTCTCCGGTACCATCGGAGGCTATTGAC-3' and 5'-CTTTCCTGATCAGTCAGC-3' for the lpd gene; and 5'-ATGGG-GACGATGCTTTGAGCGTCTGCTGTCG-3' and 5'-CCTCCACCGGC-GGCCAAGCTCTCCTACAG-3' for the bmrR gene. The bfnB gene-inducible mutant was constructed by pMutinNC integration (Morimoto et al., 2002). An internal segment of the lpd gene was PCR-amplified and ligated into pMutinNC in E. coli, and then the plasmid was integrated into the B. subtilis chromosome via a Campbell-type crossover event. PCR amplification was done with the following primers: 5'-GCCGAAAGCTTCCGCCGTTATGATCCGG- GG-3' and 5'-CGCGGATCTCAGTTAAGGTGAGGACACG-3'. After successful integration of the recombinant plasmid, the bfnB locus was placed downstream of the spc promoter (Fig. 6b, c), and we were able to control its expression with IPTG in the constructed mutant.

Preparation of ultrathin section samples and transmission electron microscopy (TEM) observation. Samples for TEM observation were prepared by the rapid freezing and substitution-fixation (RFS) method (Kishi-Nishizawa et al., 1990; Iwano et al., 1999). Cells cultured on plates or under cover glass were rapidly fixed in liquid propane cooled with liquid nitrogen (Leichert, KF80) and transferred to liquid nitrogen. Frozen materials were subsequently substituted by 2% osmium tetroxide-acetone solution at −70 °C for 2 days. Some samples were fixed with a mixture of 1% osmium tetroxide and 2.5% glutaraldehyde in phosphate buffer (pH 7-2) containing 0.1 M sucrose. The materials were dehydrated and then embedded in Spurr’s resin (TAAB Laboratories). Ultrathin sections were observed with a transmission electron microscope (Hitachi, H7100) after staining with uranyl acetate and lead citrate.

RESULTS

Change of cell shape under cover glass

Fig. 1 shows phase-contrast images taken at 2 h intervals of B. subtilis 168 growing under cover glass on a CI agar plate. The inoculated cells were bacillary and cell density became high after 4 h of cultivation. After 6–8 h, the cells started to change their shapes to partly swollen forms, and at 12 h, most of the cells showed spherical shapes. Some of these spherical cells seemed to break down after 14 h of cultivation. Fig. 2 shows optical microscopic images of shape-changed cells; coccoïd and irregular-shaped cells were observed. The cell shape change occurred first at the centre of bacterial growth under cover glass, and the cells at the edge of the growth showed little shape change.

Such cell shape change under cover glass was only observed on CI medium, not on Spizizen’s minimum medium or rich media (LB, BHI, Mycoplasma and TBAB). On the rich media, marked cell elongation was observed at the edge of the growth, but neither spherical cell shape change nor death of cells, seen in the CI medium, was observed even after prolonged culturing (2–3 days).

Proteins expressed in the shape-changed cells

Fig. 3 shows the protein profiles of the cells cultured on a CI agar plate and under cover glass. The expressed proteins differed in several ways between the two cultures. The proteins in the gel bands labelled 1–9 in Fig. 3 were analysed by LC/MS/MS. The proteins detected in only one lane are shown in bold in the table to the right of the gel. It may reflect the oxygen availability that the cells cultured on the agar plate expressed the vegetative catalase (KatA, spot O3) and the cells under cover glass expressed nitrate reductase (narG, spot U1) for nitrate respiration and the enzymes for fermentation, including z-acetolactate decarboxylase (AlsD, spot U9), z-acetolactate synthase (AlsS, spots U2 and U9) and L-lactate dehydrogenase (LctE, spots U7, U8 and U9).

Based on the observation of the difference in cell shapes between the centre and the edge of the growth, and also from the result of protein analysis, we hypothesize that one of the triggers of cell shape changes under cover glass is the lack of oxygen.

Effect of adding KNO3 to the medium

We assumed that the differences in protein expression between the cells grown on plates and under cover glass were due to distinct culture conditions, especially differences in O2 availability. To investigate the proteins involved in cell shape change in more detail, we compared the protein expression between cover glass cultures under different conditions. Adding various substances to CI or Spizizen’s medium showed that nitrate and nitrite repressed the cell shape change in the CI microcultures. Fig. 4 shows phase-contrast images of cells under cover glass cultured with or without KNO3. The addition of 0.01% KNO3 almost repressed the cell shape change (Fig. 4b) and the addition of 0.1% KNO3 repressed it completely (Fig. 4c), and also, no cell death was observed over 2 days of culture. NaN3O2 (0.01% and 0.1%) also substantially repressed the cell shape change under cover glass culture (not shown). The fact that supplementation with NO3 or NO2 repressed the cell shape change indicates that the lack of electron acceptors may be a factor in morphological change.

Fig. 5 shows the protein profile of the cells cultured under cover glass on CI and CI + 0.1% KNO3. Gel slices from both lanes, marked from 01 to 23, were cut out and subjected to LC/MS/MS analysis. In this analysis, the sensitivity of the apparatus was so high that many proteins were detected from the gels, including proteins specific to or strongly expressed in shape-changed cells or normally grown cells. Among them, it appeared that the level of branched-chain z-keto-acid dehydrogenase (BCHD) enzyme complex
Fig. 1. Time-course phase-contrast images of *B. subtilis* 168 cells grown under cover glass.
(BkdA1, BkdA2 and BkB, encoded by the bfmB gene locus) was higher in the shape-changed cells (spots 09, 10, 17, 18, 19 and 20 of lane C1 and spot 10 of lane C1 + KNO3 in Fig. 5). The BCDH complex has been characterized from several sources, including Pseudomonas aeruginosa (McCully et al., 1986), Pseudomonas putida (Sokatch et al., 1981), rabbit liver (Paxton & Harris, 1982), and rat and bovine kidneys (Odessay, 1982; Pettit et al., 1978), and gene

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**Fig. 2.** Optical microscopic image of shape-changed cells of *B. subtilis* 168 under cover glass culture (18 h of cultivation; magnification ×1200). (a, b) Spherical-shaped cells. (c) Irregular shaped cells.

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**Fig. 3.** Whole-cell protein profile of cells cultured on CI agar (‘On’) and under cover glass (‘Under’). Proteins identified by the LC/MS/MS system from nine gel spots of both lanes are shown on the right, and their descriptions can be found in Table 2. n.d., Not determined. The cells were harvested after 18 h of cultivation and the amount of protein loaded into each lane was approximately 35 µg.
Table 2. Descriptions of identified proteins in Fig. 3

<table>
<thead>
<tr>
<th>Protein</th>
<th>Mol. mass (kDa)</th>
<th>Description</th>
<th>Protein</th>
<th>Mol. mass (kDa)</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>AhpC</td>
<td>20-6</td>
<td>Alkyl hydroperoxide reductase small subunit</td>
<td>NarG</td>
<td>139-1</td>
<td>Nitrate reductase</td>
</tr>
<tr>
<td>AlsD</td>
<td>28-8</td>
<td>2-Acetolactate decarboxylase</td>
<td>OppA</td>
<td>61-4</td>
<td>Oligopeptide ABC transporter (binding protein)</td>
</tr>
<tr>
<td>AlsS</td>
<td>62-1</td>
<td>2-Acetolactate synthase</td>
<td>OxdC</td>
<td>43-4</td>
<td>Acid-induced cytosolic oxalate decarboxylase</td>
</tr>
<tr>
<td>CitC</td>
<td>46-4</td>
<td>Isocitrate dehydrogenase</td>
<td>PdhA</td>
<td>41-5</td>
<td>Pyruvate dehydrogenase</td>
</tr>
<tr>
<td>CitH</td>
<td>45-4</td>
<td>Divalent metal ions/citrate complex secondary transporter</td>
<td>PdhB</td>
<td>35-5</td>
<td>Pyruvate dehydrogenase</td>
</tr>
<tr>
<td>CitZ</td>
<td>41-6</td>
<td>Citrate synthase II</td>
<td>PdhD</td>
<td>49-7</td>
<td>2-Oxoglutarate dehydrogenase</td>
</tr>
<tr>
<td>FeuA</td>
<td>35-1</td>
<td>Iron-uptake system (binding protein)</td>
<td>Fik</td>
<td>34-3</td>
<td>6-Phosphofructokinase</td>
</tr>
<tr>
<td>FtsZ</td>
<td>40-4</td>
<td>Cell-division initiation protein (septum formation)</td>
<td>Fgk</td>
<td>42-2</td>
<td>Phosphoglycerate kinase</td>
</tr>
<tr>
<td>Gap</td>
<td>35-8</td>
<td>Glyceraldehyde-3-phosphate dehydrogenase</td>
<td>Psl</td>
<td>63-1</td>
<td>PTS enzyme I</td>
</tr>
<tr>
<td>GltX</td>
<td>55-7</td>
<td>Glutamyl-tRNA synthetase</td>
<td>ResD</td>
<td>27-5</td>
<td>Two-component response regulator</td>
</tr>
<tr>
<td>GroEL</td>
<td>57-4</td>
<td>Class I heat-shock protein</td>
<td>SerA</td>
<td>57-1</td>
<td>Phosphoglycerate dehydrogenase</td>
</tr>
<tr>
<td>GuaA</td>
<td>58-0</td>
<td>GMP synthetase</td>
<td>TasA</td>
<td>28-2</td>
<td>Translocation-dependent spore component</td>
</tr>
<tr>
<td>Hag</td>
<td>32-6</td>
<td>Flagellin protein</td>
<td>Tpi</td>
<td>27-0</td>
<td>Triose-phosphate isomerase</td>
</tr>
<tr>
<td>IlvC</td>
<td>37-5</td>
<td>Ketol-acid reductoisomerase</td>
<td>WapA</td>
<td>258-0</td>
<td>Cell-wall-associated protein precursor</td>
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<tr>
<td>KatA</td>
<td>54-6</td>
<td>Vegetative catalase 1</td>
<td>YcgT</td>
<td>36-8</td>
<td>Similar to thiorodoxin reductase</td>
</tr>
<tr>
<td>LctE</td>
<td>34-8</td>
<td>L-Lactate dehydrogenase</td>
<td>YdiL</td>
<td>37-3</td>
<td>Similar to L-iditol 2-dehydrogenase</td>
</tr>
<tr>
<td>MetI</td>
<td>41-5</td>
<td>Cystathionine γ-synthase</td>
<td>YbjI</td>
<td>39-3</td>
<td>Unknown</td>
</tr>
<tr>
<td>MntA</td>
<td>33-4</td>
<td>Manganese ABC transporter (membrane protein)</td>
<td>YqiE</td>
<td>39-6</td>
<td>Similar to tripeptidase</td>
</tr>
<tr>
<td>MreB</td>
<td>36-0</td>
<td>Cell-shape-determining protein</td>
<td>YrbB</td>
<td>40-9</td>
<td>Similar to cystathionine γ-synthase</td>
</tr>
<tr>
<td>MtnK</td>
<td>45-3</td>
<td>Methyllthioribose kinase</td>
<td>YurP</td>
<td>36-7</td>
<td>Probable phosphosugar isomerases</td>
</tr>
<tr>
<td>NadE</td>
<td>30-4</td>
<td>NH3-dependent NAD^+ synthetase</td>
<td>YurX</td>
<td>48-3</td>
<td>Unknown</td>
</tr>
</tbody>
</table>
homologues have been found in Bacillus anthracis, Bacillus stearothermophilus, Staphylococcus aureus and Streptococcus mutans (Wang et al., 1993). The BCDH complex is involved in the biosynthesis of branched-chain fatty acids (Willecke & Pardee, 1971), which are the major components of membrane fatty acids in B. subtilis (Kaneda, 1977).

Cell shape of the bfmb deletion mutant and IPTG-inducible mutant grown under cover glass

Fig. 6 shows phase-contrast images of the mutants cultured under cover glass on CI and CI + 1 mM IPTG. As shown in Fig. 6(a), the deletion of bfmb repressed the cell shape change under cover glass. In strain LPDd, the cells cultured under cover glass on CI medium showed little shape change (Fig. 6b). In this condition, the expression of four genes, lpd, bkdA1, bkdA2 and bkdB, was suppressed. On CI +1 mM IPTG, where only the expression of the gene lpd was abolished, strain LPDd did show changes in cell shape (Fig. 6c). These results suggested that the expression of bfmb genes, which code for the BCDH complex, was related to the cell shape change that occurred under cover glass culture.

TEM observation of the shape-changed cells

Fig. 7 shows ultrathin section micrographs of normally grown cells and shape-changed cells. Cells were fixed with 2.5% glutaraldehyde and 1% osmium tetroxide with 0.1 M sucrose. The cells grown on the CI agar plate had thick Gram-positive-type wall structures (Fig. 7a, b). The cells in the early stage of shape change had much thinner walls (Fig. 7c). In addition, plasmolysis of the chemically fixed shape-changed cells was observed (Fig. 7c). Fig. 7(d) shows electron micrographs of a rapidly freeze-fixed cell from a culture in the late stage of shape change. This coccoid cell also showed a thin-walled structure.
DISCUSSION

*B. subtilis* has long been viewed as a strict aerobe, but recent studies have shown that it also grows anaerobically either by utilizing nitrate or nitrite as an electron acceptor or by fermentation in the absence of electron acceptors (Nakano & Hullet, 1997; Nakano & Zuber, 1998). Various mechanisms whereby microbes sense and respond to alterations in the redox state have been reported (Bauer et al., 1999; Sawers, 1999; Semenza, 1999). *B. subtilis* can respond to changes in oxygen availability and the redox state by changing metabolic directions in favour of anaerobiosis. In this study, we observed an L-form-like cell shape change of *B. subtilis* strain 168 under a limited oxygen, moderate nutrient culture condition. Anaerobox (80% N₂, 10% H₂, 10% CO₂) culture also led to deformed cell shapes (not shown). Thus far, the cell shape change has been best observed in CI cultures under low-oxygen conditions. As shown in Fig. 4, the addition of KNO₃ to CI medium suppressed the shape change. Lowering of electron acceptors may be one of the factors triggering the conversion to cell-wall-deficient forms in *B. subtilis*.

In this study, L-form-like morphological change under cover glass was only seen in CI medium, not in minimal medium or rich media. CI medium is composed of Spizizen’s minimal salts with trace amounts of yeast extract and Casamino acids. The addition of only yeast extract or Casamino acids also made *B. subtilis* cells deform (data not shown), but the addition of 50 μg ml⁻¹ of a single amino acid or a mixture of them into Spizizen’s minimal salts did not. As shown in Fig. 3, the cells grown under cover glass on CI medium expressed nitrate reductase, which may indicate that at least a trace amount of

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Fig. 6. Gene structures and phase-contrast images of the parent strain and the mutants, cultured under cover glass. (a) ddBFMBd on CI agar. (b) LPDd on CI agar. (c) LPDd on CI agar containing 1 mM IPTG. Photographs were taken at the centre of the growth after 18 h cultivation.
nitrate was present in CI medium. However, *B. subtilis* cells cultured under cover glass on Spizizen’s medium supplemented with 0.01% or 0.1% KNO₃ did not show any shape change. Further study will be needed to clarify the conditions necessary for morphological changes in *B. subtilis* and also how the cells sense these conditions.

Protein analysis demonstrated differences in expressed proteins between bacillary cells and shape-changed cells, and a mutant of the *bkd* operon did not show the shape change in CI microculture. The products of this operon are involved in the metabolism of branched-chain amino acids and the biosynthesis of branched-chain carboxylic acids (Debarbouille et al., 1999). BkdA1, BkdA2 and BkdB, the products of the *bfmB* gene locus (the last three genes of the *bkd* operon), were frequently detected from shape-changed cells. Experiments with *bfmB* mutants (Fig. 6) showed that the inactivation of *bfmB* resulted in the repression of cell shape change, and its induction gave rise to it again. The three *bfmB* genes code for the BCDH enzyme complex, which is involved in the biosynthesis of branched-chain fatty acids, the major components of the *B. subtilis* membrane (Kaneda, 1977, 1991). The physicochemical effect of a methyl branch in a long acyl chain is similar to that of a *cis* double bond. The saturated/unsaturated fatty acid ratio of *B. subtilis* grown at 20 and 37°C did not differ markedly (Kaneda, 1977, 1991), suggesting that the branched-chain fatty acids in *B. subtilis* function to maintain membrane fluidity at low temperature. Morphological and physiological studies have indicated the importance of membrane synthesis during sporulation for asymmetric septation and prespore engulfment (Piggot et al., 1994). Bourdreaux & Freese (1981) studied the sporulation rate and the fatty acid composition of a *bfmB* mutant grown with a wide range of fatty acid precursors. They reported that the *bfmB* mutant could grow but

Fig. 7. Ultrathin section micrographs of *B. subtilis* 168. (a, b) Cells grown on a CI plate; chemically fixed. (c) Cells grown under cover glass on a CI plate, in the early stage of shape change (8 h of cultivation); chemically fixed. (d) A shape-changed cell grown under cover glass; cells in the late stage of shape change (18 h of cultivation) were freeze-fixed and freeze-substituted. Bars, 0.5 μm.
showed a 200-fold lower sporulation rate when the level of precursors is insufficient. In the present study, our bfmB mutant did not show a change in morphology to L-form-like spherical shapes. Hoischen et al. (1997) compared the fatty acid composition of Streptomyces hygroscopicus walled cells and its stable L-form, and showed that the membranes of the L-form had a higher content of branched antiso fatty acids than the membranes of the walled vegetative cells.

From our TEM observations, it appears that the cell shape change under cover glass was caused by thinning of the cell wall. Plasmolysis by 0·1 M sucrose was observed in the chemically fixed cells (Fig. 7c), as Schall et al. (1981) observed in lysozyme-treated cells of Bacillus licheniformis. The phenomena we have observed may be discussed from the aspect of the process of autolysis. To investigate whether the death of the cells observed in the later stages of microculture was due to osmotic damage or some endogeneous process, we will perform a detailed study including cultures with osmotic stabilizers.

It is not known whether the conversion to the cell-wall-deficient form is an adaptation state for some specialized environments. L-form bacteria have often been observed in or isolated from clinical specimens (Domingues & Woody, 1997; Mattman, 2001b). Mammals have developed a host defence system against micro-organisms by sensing their surface materials and recognizing them as xenobiotics. For the bacteria, removing the cell wall components may contribute to the evasion from host defence systems and the intracellular survival if they are able to live without cell wall structures.

The present study indicated that there might be a regulatory system for cell wall biosynthesis in response to environmental conditions in B. subtilis. Understanding the mechanisms and regulation of cell shape change in bacteria may lead to the development of novel antibiotics.

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